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Antibacterial, antibiofilm and viability analysis *in vitro* of calcium silicate and epoxy resin-based filling sealers

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Aim: The aim of this study was to evaluate the antibacterial and antibiofilm capacity against *Enterococcus faecalis* of endodontic sealers AH Plus Jet and the calcium silicate-based sealers, Bio-C Sealer and Sealer Plus BC. **Methods:** All experiments were performed with fresh endodontic sealers without setting. For this, antibacterial agar diffusion test, analysis of antibiofilm activity evaluated by confocal microscopy, assessment of cell viability and pH evaluation were used. **Results:** In the agar diffusion test, all sealers showed an ability to inhibit bacterial growth. In the antibiofilm analysis by confocal microscopy, all the evaluated sealers showed the ability to reduce the mature bacterial biofilm. A pH analysis was also performed in the period from 1min to 72h; calcium silicate-based sealers presented a more alkaline pH than AH Plus Jet, and Bio-C Sealer presented a pH greater than 11 in all the analyzed periods. In the assessment of cell viability AH Plus Jet demonstrated a lower cell viability after 24h. However, after 72h, all sealers led to a reduction in cell viability when compared to control. **Conclusions:** Bio-C Sealer and Sealer Plus BC and AH Plus Jet showed similar antibiofilm capacity against mature *E. faecalis* biofilms. Furthermore, all evaluated sealers showed antibacterial capacity in the agar diffusion test, and AH Plus Jet and Bio-C Sealer showed significantly greater inhibition of bacterial growth than Sealer Plus BC. Thus, evaluated sealers demonstrated antibacterial and antibiofilm activity against *E. faecalis*. Knowledge about the antibiofilm and antibacterial activity of endodontic sealers for *E. faecalis* is relevant, as it is currently one of the main causes of failure in endodontic treatment.

Keywords: Root canal filling materials. Endodontics. Root canal obturation.

Introduction

The purpose of endodontic therapy is to reduce the microbial number and load present in the root canal system and prevent subsequent reinfection[1]. After chemical-mechanical preparation, it is important to fill the root canal system. This filling aims to prevent reinfection and is performed with gutta-percha points and endodontic root sealers[2]. Endodontic sealers with different chemical compositions are available on the market. Among them, bioceramic sealers are calcium silicate-based sealers[3] which has been showing bioactive properties[4,5]. They have also been successfully used in endodontic treatments, including pulp capping, apical barrier formation, periapical defect repair and perforation sealing[5].

These sealers have adequate physicochemical[3,6], biological[7,8] and antimicrobial properties[9]. They have been demonstrated greater bioactivity and biocompatibility activities[7,8] comparing to endodontic sealers that preceded them. Their bioactivity occurs through the release of molecules that favor repair during and after setting[8]. The setting reaction of this biomaterial occurs through a hydration process. In this reaction, when calcium silicate is mixed with water, the formation of calcium silicate hydrogel and calcium hydroxide occurs, which partially react with the phosphate to form crystals of hydroxyapatite and water[9], favoring bone repair[5,7,8]. However, the high solubility of bioceramic sealer generates its bioactive potential even after the setting reaction, resulting in a higher alkaline pH[,10], which plays a positive role in apical healing, contributing to the formation of mineralized tissues[7,8] and antimicrobial effect[9]. Some studies attribute the antimicrobial activity of calcium silicate-based sealers to the release of calcium hydroxide ions during sealer setting, which leads to an increase in pH[9].

Due to its properties, some ready-to-use calcium silicate-based filling sealers have appeared on the market, such as Bio-C Sealer (Ângelus, Londrina, PR, Brazil) and Sealer Plus BC (MK Life, Porto Alegre, RS, Brazil)[7]. Bio-C sealer is composed by calcium oxide, silicon oxide, iron oxide, polyethylene glycol and tricalcium aluminate in its composition[7]. While Sealer Plus BC is composed by nanoparticulate of calcium trisilicate, zirconium oxide and calcium disilicate[7]. Differences between sealers compositions can interfere with their properties.

Endodontic treatment reduces but cannot completely eliminate microorganisms from the root canal system[9]. Among these microorganisms, *Enterococcus faecalis* is a facultative anaerobic gram-positive bacterium and is found in the oral cavity with more prevalence in persistent intraradicular infections[11]. Therefore, it is often responsible for failure in endodontic treatments. This bacterium has the ability to invade deep into the dentinal tubules, resist intracanal procedures during routine endodontic treatment, and survive in filled canals without the support of other bacteria[12]. Its survival can be explained by tolerance to antimicrobials and the ability to survive in adverse environments[13] and to form biofilms[9,13,14].

Microorganisms are established in biofilms in root canal systems infected in endodontic infections[15]. Bacteria living in biofilms are more resistant to antimicrobials compared to planktonic bacteria[15]. Consequently, the use of root canal

filling materials with antibacterial activity is considered beneficial in reducing the number of remaining microorganisms[9,15]. It is essential to evaluate the antibacterial and antibiofilm effect of endodontic sealers, in conditions that are most like those clinically found. Sealers with antibacterial activity can help to decrease the number and residual microbial load that have survived chemical mechanical instrumentation and thus improve the success rate of endodontic treatment[9]. The antimicrobial effect of filling sealers has been previously evaluated, but few studies have been performed evaluating the antimicrobial and antibiofilm activities of Sealer Plus BC and Bio-C Sealer.

Thus, knowing that new bioceramic sealers are appearing on the market, there is a need for new studies. It is extremely important to evaluate their bioactivity and biocompatibility, and antimicrobial and antibiofilm activity, in relation to the bacterium *E. faecalis*, due to its great clinical relevance in persistent infections. Then, the aim of this study was to evaluate the effect of endodontic sealers AH Plus Jet, Bio-C Sealer and Sealer Plus BC on cellular viability and antibacterial and antibiofilm effect against *E. faecalis*.

Material and Methods

Sealer preparation

Three different endodontic sealers were evaluated: AH Plus Jet (Dentsply International Inc, York, PA, USA), Sealer Plus BC (MK Life, Porto Alegre, RS, Brazil) and Bio-C Sealer (Angelus, Londrina, PR, Brazil). AH Plus Jet sealer was prepared according to the manufacturer's instructions and Sealer Plus BC and Bio-C Sealer are ready to use. Antibacterial and antibiofilm assays were performed after sealers were placed in direct contact with the *E. faecalis* culture, while sealer extracts were used to evaluate their cytotoxicity and migratory potential in periodontal ligament cells. Sealer extracts were prepared with 110 mg of each sealer (AH Plus Jet, Bio-C Sealer and Sealer Plus BC) at the bottom of a 24-well plate. After setting period, each well was covered by supplemented DMEM medium (Sigma) or Brain Heart Infusion medium (BHI), for 24 hours at 37 °C in a humidified atmosphere with 5% CO_2. Supernatant from this preparation was filtered through a 0.20 mm-pore (Minisart; Sartorius Stedim Biotech, Göttingen, Niedersachsen, Germany)[5], for extract quality. Filtration is important to ensure that there are no large sealer fragments in the extract, which could influence cell viability.

Antimicrobial and antibiofilm assays

Agar diffusion test

Microbiological assays were carried out under aseptic conditions in a laminar flow chamber (Quimis, Diadema, SP, Brazil), following Candeiro et al.[16] (2016) with adaptations. Antibacterial activity was evaluated using the *E. faecalis* strain (ATCC 29212). Microorganisms were cultivated in Mueller-Hinton agar (Kasvi, São José dos Pinhais, PR, Brazil) at 37 °C, for 24h. Then, three colonies were selected and incubated in 5 mL of Mueller-Hinton broth at 37 ºC, 220 rpm, for one night.

Subsequently, 15 µL of pre-inoculum was added to each 5 mL of Mueller-Hinton medium and incubated at 37 ºC, at 220 rpm. This inoculum was read by optical density (O.D.) every hour, until reaching a reading of 0.100, corresponding to $3x10^8$ CFU.mL^{-1}. A swab was used to spread bacteria on five plates containing Mueller-Hinton agar. Afterwards, three wells of 6 mm in diameter and 4 mm in depth were made with a tip, removing the agar at equidistant points and immediately filling wells with the materials to be evaluated. Negative control was represented by the absence of sealer in culture and positive control, by culture containing ampicillin (100 µg). All plates were incubated at 37 °C for 72h under aerobic conditions and in a humid environment. Zones of inhibition around each well were then measured in millimetres using a specimeter.

Antibiofilm Activity Tests

The methodology used here was previously described by Silva et al.[17] (2021) and was carried out with adaptations. From each bovine tooth, 2 dentin discs were produced, and all adjacent enamel was removed. A total of twelve bovine teeth were used. Bovine teeth crowns were cut, and dentin discs of 1 mm thick and 4 mm in diameter were performed[14]. Discs were treated with 0.5 M EDTA for 60 s on both sides and then washed with abundant distilled water[18]. Afterwards, discs were individually wrapped and sterilized in an autoclave (saturated steam under pressure). Then, discs were inserted into the bottom of a 24-well plate, followed by bacterial suspension of *E. faecalis* in Mueller-Hinton medium at 10/990 v/v per well. Plates were incubated at 37 ºC for 15 days, with medium replacement every 3 days. After the time elapsed, dentin discs were gently washed with PBS and then inserted into a new 24-well plate, lined with 110 mg of the tested sealer[16]. Biofilm was exposed to filling sealers based on calcium silicate and AH Plus Jet sealer for 5 days. *E. faecalis* bacteria in Mueller-Hinton medium represented positive control, while negative control was represented by *E. faecalis* culture containing 100 µg.mL^{-1} ampicillin (Sigma-Aldrich, St Louis, USA). After 5 days, discs were washed in PBS twice to remove culture medium and non-adherent cells. Then, disc surface was stained with 50 µL of live/dead baclight bacterial viability kit composed of SYTO 9 and propidium iodide (Thermo Fisher Scientific, Waltham, MA, USA), incubated at room temperature, for 10 minutes, and observed in an inverted confocal laser scanning microscope (Leica TCS-SPE; Leica Biosystems CMS, Mannheim, Baden-Württemberg, Germany). Six image captures were taken of each sample with a 40x magnification. Each image was representative of a 387.5 x 387.5 µm^2 field. Images were then transferred to the Imaris 7.2 software (Bitplane Inc, St Paul, MN, USA). A biofilm analysis tool was used to evaluate the 8 fields of each sample. Antibiofilm assay was performed in three replicates of individuals, conducted on different days. Results for each group generated a single mean, representative of 8 fields from three biological samples. Total biovolume of *E. faecalis* live cells (µm^3) in dentin discs after 15 days of matured biofilm formation was analysed followed by sealers exposure for 5 days, evaluated by confocal microscopy. Biofilm eradication was calculated according to 3 image captures for each independent replica. Data were presented as mean and standard error os replicata. Control was represented by *E. faecalis* in Mueller-Hinton medium and statistical differences were considered when $p < 0.05$.

pH evaluation

Sealers were prepared according to the manufacturers' instructions and were inserted into the bottom of a 24-well plate. Then, 120 mg of each sealer was inserted into each well and this experiment was performed in duplicate. After inserting the sealers, an amount of distilled water was added to cover ¾ of each well. pH values were measured at 1, 5, 15, 30 and 60 minutes, 24, 48 and 72 hours after the addition of water using a temperature compensated electrode with a pH meter (SB70P; VWR, West Chester, PA, USA). This methodology had been previously described by Zhang et al.[9] (2009) and here was carried out with adaptations.

Periodontal ligament cell culture assays

Periodontal ligaments were obtained from intact third molars extracted from 4 adult patients aged between 18 and 30 years, after approval by the Human Research Ethics Committee of Faculdade São Leopoldo Mandic (protocol number 52211421.0.0000.5374). After tooth extraction, root scaling was performed using periodontal curettes, and tissue structures of the periodontal ligament were collected[19]. Periodontal ligament tissue obtained was immersed in a solution containing DMEM (Modified Eagle's Medium, Sigma Chemical) with fetal bovine serum, and fixed in a cell culture plate[20]. Then, periodontal ligament product was cultured in 6-well plates (Corning®, Corning, NY, USA), in DMEM culture medium containing 10% fetal bovine serum (FBS) (Cultilab, Campinas, São Paulo, Brazil), 100 $IU.mL^{-1}$ of penicillin (Invitrogen, Waltham, MA, USA), 100 $\mu g.mL^{-1}$ of streptomycin (Invitrogen) and 2 $mmol.L^{-1}$ of glutamine (GIBCO, Dublin, Leinster, Ireland), in a humidified atmosphere containing 5% CO_2, at 37 ºC. These cells were subcultured every three days and used between 3 and 6 passages[19]. For the MTT and cell migration tests, extracts from the above-mentioned sealers were used.

Cell viability assay

The cell viability assay was carried out after the period of 24 and 72h, by MTT assay (Sigma). Negative control was represented by cells in culture medium, and positive control, by cells in lysis solution (10 Mm Tris, pH 7.4, 1 Mm EDTA and 0.1% triton X-100). An additional group was performed, represented by an extract of AH Plus (1:1) – gold standard. After the experimental period, cell culture medium was removed, and 100 µL of DMEM containing 10 µL of MTT (5 $mg.mL^{-1}$) solution (Sigma), was added. Plates were incubated in a humidified oven at 37 °C, for a period of four hours. Then, 100 µL of dimethylsulfoxide – 100% DMSO was added, and the plate was read at 570 nm[21].

Statistical analysis

Data were taken with GraphPad Prism® version 10 (GraphPad Software, Inc., San Diego, California, USA). Statistical analysis of the data and frequency distribution was generated for all study variables. Cell viability and cell migration data were verified by the Kolmogorov-Smirnov test. If data were updated in a standard, the data were shifted as mean and standard deviation (SD). One-way ANOVA test was used. Statis-

tical differences in the analysis of antibacterial and antibiofilm tests were verified by Tukey's one-way ANOVA post-test. The significance value was considered $p < 0.05$.

Results

Antibacterial disk diffusion analysis

Tested endodontic sealers were able to inhibit the growth of *E. faecalis* (ATCC 29212) in agar diffusion test. In all analyzed periods, all sealers produced a halo of bacterial inhibition that was smaller than that of the positive control group. AH Plus Jet provided the greatest inhibition of *E. faecalis* at all evaluated periods. Sealer Plus BC showed a statistically lower bacterial inhibition halo than that observed in the presence of AH Plus Jet, in all analyzed periods. The bacterial inhibition halo in the presence of Bio-C Sealer was similar to what was observed in the presence of AH Plus Jet, at 24h and 48h. However, at 72h, this inhibition halo was statistically lower than that observed in the presence of AH Plus. In addition, Sealer Plus BC at 48h also showed a lower capacity to inhibit bacterial growth, when compared to the Bio-C Sealer (Table 1).

Table 1. Agar diffusion test. The size of the inhibition zones was measured in all groups (Control, AH Plus Jet, Bio-C Sealer and Sealer Plus BC). Control group was represented by Ampicillin 100 $\mu g.mL^{-1}$. Comparisons between groups were performed with averages from analyses within the same experimental period (0h, 24h and 48h). Significance value was considered when $p < 0.05$, comparing to control (*), AH Plus Jet (#), and Bio-C Sealer (□), by one-way ANOVA and Tukey's post-test.

Group	24h	48h	72h
Control	4.983 ± 1.45	4.611 ± 1.05	4.178 ± 1.01
AH Plus Jet	3.928 ± 0.50 *	3.43 ± 0.46 *	3.300 ± 0.46 *
Bio-C Sealer	3.411 ± 0.63 *	3.028 ± 1.02 *	1.617 ± 0.32 *#
Sealer Plus BC	2.711 ± 1.12 *#	1.933 ± 0.43 *#□	1.239 ± 0.22 *#

Antibiofilm activity of AH Plus Jet, Bio-C Sealer and BC Sealer against *E. faecalis*

Confocal microscopy analysis demonstrated a similar antibiofilm capacity of endodontic sealers based on calcium silicate and epoxy resin to reduce mature biofilms grown on dentin discs of bovine teeth. However, all tested sealers did not eradicate mature biofilm of *E. faecalis* (ATCC 29212) (Figure 1 and 2). All analyzed sealers (Figure 1-B, 1-C and 1-D) were able to kill more bacterial cells in *E. faecalis* biofilm, thus reducing the biovolume of bacterial cells compared to the control group (Figure 1-A). The control group (Figure 1-A) has a higher biovolume of live cells (green) than the other groups, as can be seen in Figure 2.

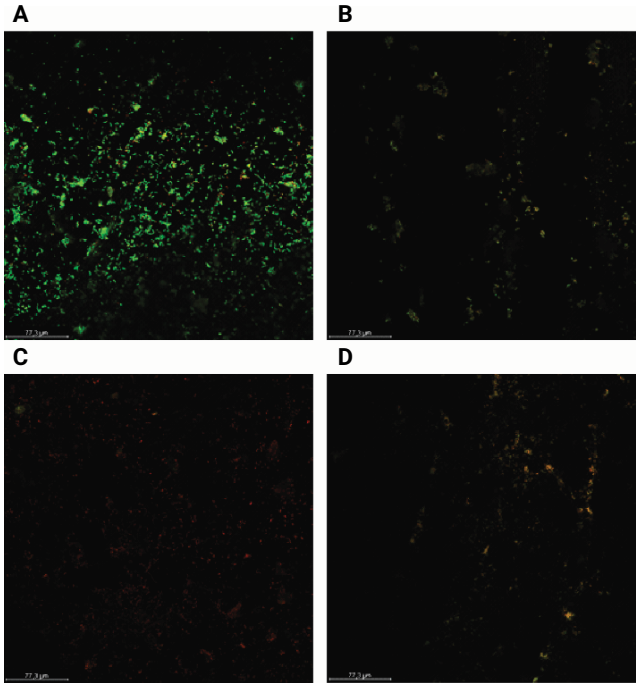

Figure 1. Confocal microscopy images of Control (A), AH Plus Jet (B), Bio-C Sealer (C) and Sealer Plus BC (D) groups. Biofilm was stained with live/dead baclight bacterial viability kit composed of SYTO 9 (which stains live bacteria green) and propidium iodide (which stains dead bacteria red). Scale bar: 77.3 µm.

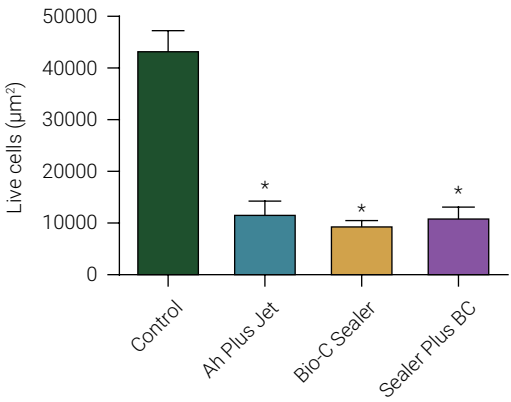

Figure 2. Biovolume graph confocal microscopy. Total biovolume of *E. faecalis* live cells (µm³) (E) in dentin discs after 15 days of matured biofilm formation followed by sealers exposure for 5 days, evaluated by confocal microscopy. Biofilm eradication was calculated according to 3 image captures for each independent replica. Representation of three independent replicas. Data were presented as mean ± standard error. * $p < 0.05$ compared to control, by one-way ANOVA and Tukey's post-test. Control was represented by *E. faecalis* in Muller Hinton medium.

Evaluation of sealers' pH

AH Plus Jet had a lower pH at all tested periods, with a progressive reduction in its pH as the time was increased. A reduction in pH was also evaluated in the presence

of Sealer Plus BC, mainly after 24h. Bio-C Sealer showed pH stability up to the final period evaluated, corresponding to 72h. There was a statistical difference between sealers based on calcium silicate and AH Plus, in all evaluated periods. There was also a statistical difference in pH between calcium silicate-based sealers (Bio-C Sealer and Sealer Plus BC) at 15 min, 24h, 48h and 72h (Table 2).

Table 2. pH assessment of AH Plus, Bio-C Sealer and Sealer Plus BC sealers. Evaluation of sealers' pH after 1 min, 5 min, 15 min, 30 min, 1h, 24h, 48h and 72h. Statistical analyzes were performed comparing AH Plus Jet (*), and Bio-C Sealer (#), in each experimental period by Tukey's one-way post-test ANOVA, p <0.05.

Group	1 min	5 min	15 min	30 min	1h	24h	48h	72h
AH Plus Jet	10.641± 0.249	10.506 ± 0.199	10.671 ± 0.069	10.571 ± 0.135	10.538 ± 0.053	7.74 ± 0.449	6.87 ± 0.21	6.8 ± 0.085
Bio-C Sealer	11.498 ± 0.186*	11.368 ± 0.061*	11.32 ± 0.213*	11.493 ± 0.182 *	11.563 ± 0.123*	11.51 ± 0.171*	11.635 ± 0.266*	11.735 ± 0.147*
Sealer Plus BC	11.4833 ± 0.168*	11.58 ± 0.141*	11.596 ± 0.100*#	11.601 ± 0.084*	11.633 ± 0.054*	10.723 ± 0.145*#	9.351 ± 0.082*#	8.553 ± 0.169*#

Cell viability assay

After 24h, AH Plus Jet sealer demonstrated a lower cell viability compared to control and to calcium silicate-based sealers, Sealer Plus BC and Bio-C Sealer (Figure 2). However, after 72h, all sealers led to a cell viability reduction when compared to control. Therefore, Sealer Plus BC led to a lower cell viability when compared to AH Plus Jet sealer (Figure 3).

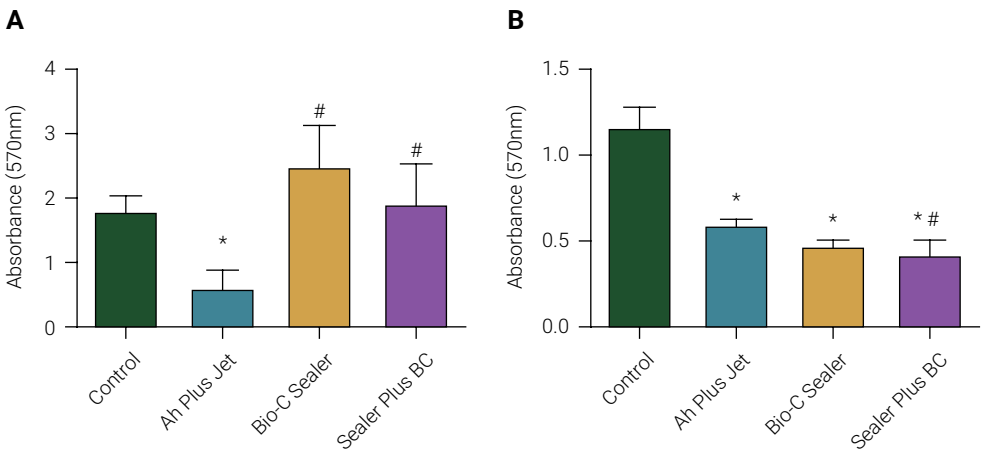

Figure 3. MTT assay in periodontal ligament cells after sealers extracts (1:1) exposure during 24h (A) and 72h (B). In 24h(A), the AH Plus Jet cement had lower cell viability compared to the control(*) and also had less cell viability compared to the calcium silicate-based cements Sealer Plus BC and Bio-C Sealer(#). However, after 72h(B), all sealers had reduced cell viability when compared to the control group(*). Therefore, the Sealer Plus BC sealer had lower viability when compared to the AH Plus Jet sealer(#) (B). Data were presented as mean ± standard error. * p < 0.05 in relation to the control, by oneway ANOVA and Tukey's post-test. Control was represented by cells in DMEM medium.

Discussion

Root canal cleaning and shaping reduce the amount and virulence of microorganisms in an infected root canal. However, it is not possible to eliminate microorganisms from the entire root canal system[15]. Thus, the use of sealers containing antibacterial properties is considered beneficial in reducing the number of remaining microorganisms[9]. The present study evaluated the antibacterial capacity of calcium silicate-based sealers and AH Plus Jet against *E. faecalis*. This bacterium is an important species related to persistent endodontic infections[1,9,11]. Its pathogenic capacity is probably due to its ability to survive and persist in diverse conditions, such as high pH[13] and, its ability to form biofilm[9,13,14]. Hence, this bacterial species has been used to evaluate antibacterial properties of different endodontic sealers[1,9,15,22-27].

Among commercially available sealers, AH Plus Jet is an epoxy resin-based one, considered the gold standard due to its high strength bonding to dentin, radiopacity, performance, dimensional stability, low solubility, and high overall strength[3]. Previous studies demonstrated cytotoxicity in the presence of this material[8,28]. Furthermore, this sealer does not resorb easily when extravasated into the periapical tissues, which can generate a short-term local inflammatory process[23]. Jung et al.[28] (2018) in an *in vitro* study, demonstrated high cytotoxicity of AH Plus at a 1:10 concentration of freshly mixed AH Plus extract in human osteoblastic cell culture, up to 21 days. Surviving cells were only observed when AH Plus was diluted at 1:100 and after setting. This sealer's cytotoxicity may occur by the release of formaldehyde in combination with the release of amine components and epoxy resin during the sealer setting [9,28], which can lead to a delay in tissue repair[29].

Nowadays, a new class of endodontic sealers has emerged, namely the calcium silicate-based sealers. Several previous studies compared the properties of calcium silicate-based sealers to other already established sealers, such as AH Plus Jet. These results demonstrated that calcium silicate-based sealers presented good physicochemical properties such as apical sealing, even better than AH Plus, a fact that is probably correlated to its hydrophilic property which allows a more appropriate sealing in the apical region[30]. According to previous studies, sealers based on calcium silicate have a higher flow rate than AH Plus[10] and generate a better seal between dentin and filling material, as they have smaller molecules and are hydrophilic, being able to imbricate more deeply in the dentinal tubules[6]. Other characteristics of sealers based on calcium silicate correspond to high solubility in tissues, being reabsorbed when extravasated and observed to be greater than AH Plus[10]. The low solubility of AH Plus can be attributed to the strong chemical cross-links that this epoxy resin-based material presents[10], in addition to its high strength bonding to dentin[31]. However, the high solubility of the bioceramic sealer generates its bioactive potential even after the setting reaction, resulting in a more alkaline pH[3,8,10], which agrees with the result found in this study, in which calcium silicate-based sealer had a higher pH than AH Plus Jet, at all tested periods. As the pH of AH Plus Jet is alkaline, before setting and close to neutral after its setting period, this finding agrees with previous studies[3,9,10].

The present study evaluated cell viability in periodontal ligament cell culture by MTT assay. Cells were exposed to sealer extract at a concentration of 1:1, for 24h. AH Plus Jet exposed cells presented reduced cell viability compared to other sealers groups. After 72h, there was a reduction in cell viability in the presence of all sealers, when compared to the control. Zordan-Bronzel et al.[22] (2021) obtained a similar result in a human osteoblastic cell culture, exposed to AH Plus Jet, Total Fill BC and Sealer Plus BC extracts at concentrations of 1:1 and 1:2. After 24h, Sealer Plus BC reduced cell viability, compared to other tested sealers and control. At 1:8 dilution, similar cell viability between sealers at 24h and 72h was observed. However, Sealer Plus BC demonstrated greater cell viability at 7 days. Ferreira et al.[32] (2022) analysed a similar concentration of sealer extracts (1:10) in apical papilla cells, for 72h. All sealers led to similar cell viability as the control after 24h. Sealer Plus and Sealer Plus BC led to a higher viability comparing to AH Plus Jet and MTA Fillapex, after 72h. Differences in viability between the aforementioned studies may be related to the use of different sealer extract concentrations and different culture cell types.

Calcium silicate-based sealers presented the highest alkaline pH compared to others and this level was maintained over 72 hours. Previous studies attributed the positive role of these sealers in the apical healing[7-8] and antimicrobial properties[9] to this alkaline pH and to the release of calcium ions[3]. Alkaline pH above 11 can eliminate *E. faecalis* and other bacteria related to periapical inflammation induction or maintenance[33]. Bio-C Sealer presented a pH above 11 at all analysed periods, in this study. Giacomino et al.[8] (2019) concluded that calcium silicate-based sealers appeared to favour survival, differentiation, and osteoblastic function, which are important for bone repair. Alves Silva et al.[7] (2020) found positive markers of osteocalcin in the presence of Bio-C Sealer and Sealer Plus BC sealers in an *in vivo* study. The same was not found in the presence of AH Plus. In addition, sealers based on calcium silicate showed a faster reduction in the inflammatory process than AH Plus.

In the present study, all evaluated sealers showed antibacterial and antibiofilm properties against *E. faecalis*. All evaluated sealers were able to inhibit the bacterial growth of *E. faecalis* up to 72 h. A higher inhibition of bacterial growth was observed in the presence of AH Plus Jet and Bio-C Sealer, comparing to what was observed in the presence of Sealer Plus BC. Similar results were found by Candeiro et al.[16] (2016), demonstrating that AH Plus Jet generated a halo of bacterial growth inhibition for *E. faecalis* that was greater than that observed in the presence of bioceramic sealer. As in our study, Barbosa et al.[23] (2020) used the agar diffusion test as the most basic antimicrobial method, with the aim of performing an initial analysis of the sealers. A similar capacity to inhibit the growth of *E. faecalis* between Bio-C Sealer, AH Plus, Endosequence BC Sealer, Bio-C Sealer, Sealer 26 (Dentsply International Inc, York, PA, USA) and Sealapex (Sybron Kerr, Romulus, MI, USA) was found in this study[16].

According to Guerreiro-Tanomaru et al.[14] (2013), many microorganisms are recognized for their ability to adhere, colonize, and form biofilms on the surface of root canal dentin and apical cement. Biofilm growth is an adaptive process that allows

microorganisms to survive adverse conditions[13]. Biofilm provides structural features that allow efficient transfer of nutrients, removal of waste materials and circulation of secondary metabolites and pheromones, in addition to genetic exchanges, which can make this microbiota more resistant to antimicrobials[13].

Mature biofilm of *E. faecalis* was used in this study because biofilm of this bacterium is poorly structured during the first few days[14]. In addition, the mature biofilm is closer to what was observed in the clinical environment, and one of the factors that determine biofilm resistance is its stage of development, making it more resistant to antibiotics[14,24].

The present study demonstrated that the epoxy resin sealer AH Plus Jet and the calcium silicate sealers Bio-C Sealer and Sealer Plus BC were able to reduce biofilm of *E. faecalis*, but not to eradicate it, and that they all have similar antibiofilm capacity. A previous study also observed similar results, demonstrating that AH Plus sealer and bioceramic sealer Endosequence BC Sealer, have antibiofilm activity at days 1, 7 and 30[25]. Alsubait et al.[15] (2019) performed the *E. faecalis* antibiofilm analysis of the AH Plus, BioRoot RCS, and Totalfill BC sealers (FKG Dentaire SA, La Chaux-de-Fonds, Switzerland) and the biofilm was analysed on days 1, 7 and 30, and on day 1 there was no significant difference between the analysed sealers. Results of this study showed that all sealers killed significantly more bacteria than the group without sealer after 30 days, corroborating our study[15]. The calcium silicate-based sealers in this study had antibiofilm properties, and the antibiofilm of BioRoot RCS sealer is significantly higher than that observed in the presence of Totalfill BC and AH Plus sealer after 30 days[15]. Rezende, et al.[26] (2016) carried out a study with another biofilm analysis methodology, which observed a result similar to what was found in the present study, in which both AH Plus and Sealapex bioceramic sealer reduced *E. faecalis* biofilm but were not capable of eradicating it.

However, different results from the present study are also observed in the literature. Bukhari and Karabucak[24] (2019) observed that Endosequence BC Sealer was able to reduce *E. faecalis* biofilm significantly when compared to AH Plus after a confocal microscopy analysis of 24h and 13 days of culture. Studies also demonstrate that Bio-C Sealer[27] and Sealer Plus BC[22,27] presented a greater reduction in *E. faecalis* biofilm than AH Plus sealer, using the crystal violet assay. Differences in each methodology used may be responsible for differences in the results found. Confocal microscopy was the chosen methodology for this study, based on several other studies in the literature[15,24,25].

Furthermore, dentin slices were used to develop a sparse infection[25], and to promote microbial interaction at this interface[14]. It has been known that different substrates can interfere with the results[14]. Studies attribute the antimicrobial activity of calcium silicate-based sealers to the release of calcium hydroxide ions and the increase in pH generated in the setting of the material[9,15]. For this reason, even though AH Plus has the lowest pH among the evaluated sealers, its antimicrobial effect is possibly due to the release of formaldehyde, amine components and epoxy resin during the polymerization process[9,28]. Due to the release of these molecules, AH Plus Jet maintained its antibacterial and antibiofilm effect despite its reduced pH after setting. *E. faecalis*, which was evaluated in this study, is the main cause of failure in endodontic

treatment. The limitation of this study is that it is an *in vitro* study. Despite using a microorganism prevalent in failures and a primary culture cell, they are analysed *in vitro*. While we know that the microorganism-sealer and sealer-cell interactions in the *in vivo* environment are much more complex.

In conclusion, all evaluated sealers present antibacterial and antibiofilm properties against *E. faecalis*, while demonstrated a moderate toxicity in periodontal ligament cells. These little-known properties of these sealers may generate benefits for clinical use, such as promoting periapical repair, bone formation, and reducing failure rates of endodontic therapy.

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Conflict of Interest

The authors have no conflict of interest to disclose.

Data availability

Datasets related to this article will be available to the corresponding author upon request.

Author Contribution

Larissa Caroline Müller: First author, responsible for tooth collection, methodology development, and manuscript writing.

Poliana Amanda Oliveira Silva: Responsible for tooth collection, methodology development, and manuscript writing.

Cristiano Castro Lacorte: Responsible for assisting in the development of the biofilm eradication methodology.

Mauricio Gonçalves da Costa Sousa: Responsible for assisting in the development of the biofilm cultivation methodology.

Taia Maria Berto Rezende: Responsible for data analysis, financial support, and manuscript preparation.

All authors actively revised and approved the final version of the manuscript.

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